ed hosts, so long as the tumor cells were viable in the foreign host.

3. XYZ factors have thus far been proven for only 4 transplanted neoplasms of mice and rabbits all of which are Grade III-IV by histologic grading. No such factors have been observed in adult tissues, leukemias, benign and low grade tumors.

Received July 27, 1949. P.S.E.B.M., 1949, **72.**

In vitro Determination of Bacterial Sensitivity to Aureomycin. (17340)

PAUL M. BEIGELMAN (Introduced by L. A. Rantz.)

From Department of Medicine, Stanford University School of Medicine, San Francisco. Calif.

Results of aureomycin sensitivity studies of various microorganisms have deviated greatly from one another as reported by different laboratories. This has been best exemplified by wide divergences in the reported sensitivities of staphylococci to this agent. Paine and associates[1] employing both tube dilution and plate methods, noted that 22 strains of *S. aureus* required 1 to 2 μg of aureomycin per ml for inhibition. The 5 remaining strains were inhibited by higher concentrations of the antibiotic, ranging up to 12.5 μg per ml. The type of media used was not described. Price and collaborators[2] reported *S. aureus* to be inhibited by a range of .09 to 25 μg of aureomycin per ml. The determinations were made following incubation of the organisms in penicillin assay broth for 24 hours. Bryer and associates[3] stated that 0.6 μg of aureomycin per ml inhibited staphylococci. Their report contained no statement about the method of determination. Twenty-one strains of this group of organisms tested by Lankford and Lacy[4] were inhibited by .05 to 0.2 μg of aureomycin per ml. Bacto-tryptose agar was the medium employed. With a tryptose broth turbidimetric method and 50% inhibition of growth in 18 hours as the end-point, the most sensitive strain was inhibited by .012 μg of aureomycin per ml and the most resistant by .033 μg per ml.

This study is an investigation of possible causes for discrepancies in the results obtained by others. In addition, a search has been made for a simple and reliable method of determining bacterial sensitivity to aureomycin.

Methods. Growth curves of *S. aureus,* enterococcus, *S. panama,* and *E. coli* were determined by a turbidimetric method. 0.1 ml inocula of 18 hour broth cultures were added to 18 mm test tubes containing 10 ml of tryptose broth (Difco). Aureomycin crystals were dissolved in cold sterile distilled water, diluted in tryptose broth, and added to the system immediately.* The final concentrations were .01, .02, .05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg per ml. A control tube without aureomycin was included in each determination. The cultures were incubated at 37°C and the turbidity was measured in a Coleman Junior spectophotometer with a 650 m filter. Values of less than 0.1 unit of optical density indicated marked bacteriostasis and were chosen as end-points.

Tryptone glucose extract agar with para-aminobenzoic acid (Difco) was the medium used for the plate method. Aureomycin was incorporated in this medium in final concentrations identical with those described above plus 20 and 50 μg per ml, and 18 hour broth cultures were streaked on the plates. Readings were made at various intervals of time,

[1] Paine, T. F., Collins, H. S., and Finland, M., *J. Bact.,* 1948, **56**, 489.

[2] Price, C. W., Randall, W. A., and Welch, H., *Ann. New York Acad. Sci.,* 1948, **51**, 211.

[3] Bryer, M. S., Schoenbach, E. B., Chandler, C. A., Bliss, E. A., and Long, P. H., *J.A.M.A.,* 1948, **138**, 117.

[4] Lankford, C. E., and Lacy, H., *Texas Rep. Biol. Med.,* 1949, **7**, 111.

* Solutions of aureomycin in water retained full potency if stored in the frozen state.

TABLE I.
Turbidimetric Method. (Concentration of aureomycin in μg per ml).

	Tryptose broth—hr						Tryptose broth plus 10% serum—hr					
	4	14	20	40	50	60	4	14	20	40	50	60
S. aureus (41552)	.02–.05	.1–.2	.2–.5	.5	—	1–5	.05	.2–.5	.5	—	—	5
S. aureus (37215)	.05	2	—	—	.5	—	.05	.5	—	—	5	—
Enterococcus (40056)	.1	—	.5	—	5	—	.2	—	2	—	10	—
Sal. panama (40615)	.5	1–2	2–5	10	—	—	.5	2	5	10 or more		
E. coli (41010)	.5	1	2	—	10	—	.5–1	1	5	—	—	—

The figures represent the concentration of aureomycin in μg per ml, required to inhibit growth.

complete inhibition of growth being the end-point.

A number of sensitivity determinations were made with pooled sterile human sera or whole horse blood added to the basal media. Two percent hemolyzed horse blood was used for the turbidimetric method. The blood added to tryptone glucose extract agar invariably hemolyzed.

The one precaution to be consistently observed is rapid preparation and inoculation of the aureomycin media. The whole task should be performed within a few hours due to rapid deterioration of this antibiotic.

Results. Inhibitory levels of aureomycin for 2 strains of *S. aureus* as determined by the turbidimetric method were .02 to .05 μg per ml after 4 hours of incubation, 0.1 to 0.2 μg per ml after 14 hours, 0.2 to 0.5 μg per ml after 20 hours, 0.5 μg per ml in 40 to 50 hours, and 1 to 5 μg per ml after 60 hours (See Table I). A composite growth curve derived from 9 experiments is presented in Fig. 1. It demonstrates partial inhibition for *S. aureus* by .01 and .02 μg of aureomycin per ml, complete inhibition for 7 hours by .05 μg per ml, and failure of this and higher concentrations to inhibit bacterial growth as the period of incubation is lengthened. One strain of enterococcus was inhibited by 0.1 μg per ml at 4 hours, 0.5 μg per ml at 20 hours, and 5 μg per ml at 50 hours. *Salmonella panama* was inhibited by 0.5 μg per ml at 4 hours, 1 to 2 μg per ml at 14 hours, 2 to 5 μg per ml at 20 hours, and 10 μg per ml at 40 hours. For one strain of *E. coli,* the inhibitory levels were 0.5 μg per ml at 4 hours, 1 μg per ml at 14 hours, 2 μg per ml at 20 hours, and 10 μg per ml at 50 hours. With serum present in 10% concentration, the levels were the same or somewhat higher. Increases were usually to the extent of one tube dilution, but were occasionally 5 to 10-fold (3 to 4 tube dilutions) following long periods of incubation.

Subculture of the media in the tubes containing 10 μg of aureomycin per ml invariably revealed a few viable organisms. *S. aureus* so isolated was inhibited by .02 μg of aureomycin per ml at 4 hours and 0.1 μg per ml at 20 hours. For *Sal. panama,* the values were 0.5 μg per ml at 4 hours and 2 μg per ml at 14 hours. These values were comparable to those obtained with the parent strains.

Aureomycin incubated for 18 hours at 37°C was tested against *S. aureus* and *Sal. panama.* The inhibitory levels for *S. aureus* were 0.2 μg per ml at 4 hours and 1 μg per ml at 20 hours. Pre-incubation levels were .02 μg per ml and 0.2 μg per ml at 4 and 20 hours respectively. Two micrograms per ml at 4 hours and 5 to 10 μg per ml at 20 hours were required by *Sal. panama* as compared with control levels of 0.5 μg per ml at 4 hours and 2 μg per ml at 20 hours. This represents about 60 to 90% deterioration of aureomycin in 18 hours.

The aureomycin inhibitory levels observed following incubation of *S. aureus* and *Sal. panama* for 4 hours in tryptose broth containing 2% horse blood were .05 μg and 0.5 μg per ml respectively. These values corresponded closely to those obtained with the simple tryptose broth medium.

Aureomycin was added to cultures of *S. aureus, E. coli,* and enterococcus one or 2 hours after growth commenced. Further growth was inhibited if aureomycin levels of

PLATE II.
Plate Method.

Hours	Tryptone glucose extract agar-PABA					Tryptone glucose extract agar-PABA Serum					Tryptone glucose extract agar-PABA Blood				
	12	18	24	36	60	12	18	24	36	60	12	18	24	36	60
S. aureus (41552)	.05–.2	.1–.5	.5	.5	1–20	.5–1	1–2	2	5–10	10–50	.5–1	1–2	2	5	20
S. aureus (42044)	.05–.1	.1–.5	.2–.5	.5–1	1–2	.5–1	1–2	2	5	5–20	.5–1	2	2	5	20
Enterococcus (44962)	.1–.5	.5	.5–1	1–2	10–20	.5–1	1–2	2	5–10	10–over 50	.5–1	1–2	2–5	10	20–50
Sal. panama (40615)	1–2	1–2	2–5	5–10	10–20	2–5	2–10	10	5–20	10–over 50	5–10	10	10–20	20–50	50–over 50
E. coli (41010)	1–2	2	5	5–20	5–50	2	2–10	10	5–over 50	10–over 50	2–5	5	10	50	Over 50
S. aureus (37215)	.05–.2	.1–.2	.1–.2		.1–.2										
Enterococcus (40056)	.1–.2	.2–1		.5–1	1–2						.5–1	1–2	2–5	10	20–50

The figures represent the concentrations of aureomycin in μg per ml, required to inhibit growth.

the magnitude described above as inhibitory were present. No lysis was evident even with concentrations of the agent one hundred times greater than those sufficient for bacteriostasis.

The common ranges of aureomycin sensitivity determined by the plate method for 6 strains of *S. aureus* were .05 to 0.2 μg per ml at 12 hours, 0.1 to 0.5 μg per ml at 18 hours, 0.5 to 1 μg per ml at 24 hours, 0.5 to 5 μg per ml at 36 hours, and 1 to 20 μg per ml at 60 hours (See Tables II and III). The values for 5 strains of enterococcus were 0.1 to 0.5 μg per ml at 12 hours, 0.2 to 1 μg per ml at 18 hours, 0.5 to 1 μg per ml at 24 hours, 0.5 to 2 μg per ml at 36 hours, and 1 to 20 μg per ml at 60 hours. Aureomycin inhibitory levels for *Sal. panama* were 1 to 2 μg per ml at 12 and 18 hours, 2 to 5 μg per ml at 24 hours, 5 to 10 μg per ml at 36 hours, and 10 to 20 μg per ml at 60 hours. The *E. coli* values were about the same as those for *Sal. panama* Considerably higher levels were required for inhibition when serum or blood was present. At 18 hours, for example, these ranged from 2 to 20 times greater for the gram positive cocci, and 1 to 10 times increase in aureomycin concentration for the gram negative bacilli.

Four strains of *S. aureus* had been tested against penicillin and streptomycin. Three were penicillin-resistant and one was streptomycin-resistant. All strains were equally sensitive to aureomycin.

Discussion. The data presented in this study are in close agreement with those of Lankford and Lacy.[4] Very low concentrations of aureomycin inhibited *S. aureus* if the medium used was plain tryptose broth or tryptone glucose extract agar. The same was true of other organisms tested. Lowest values were obtained with the 4 hour turbidimetric method. Addition of serum or blood to the medium increased the concentration of aureomycin required for inhibition, especially when incubation was prolonged. This effect was quite marked with the plate method. The absence of bactericidal action by aureomycin and the rapid deterioration of this agent during incubation offer an adequate explanation of these observations. A richer medium would be expected to accelerate bacterial growth

TABLE III.
Aureomycin Sensitivities in μg per ml. (Tryptone glucose extract agar plate method).

Organism	Hr			
	12	18	24	36
S. aureus (Strains 1, 44488, 44381)	0.2	0.5	0.5	5
Enterococcus (40156)	0.1	—	—	1
Enterococcus (43284)	0.1	0.2	—	—
Enterococcus (45145)	0.2	0.2	0.5	—
E. coli (40382)	1	1–2	—	5
E. coli (44532)	1	5	5	20
E. coli (43768)	1	2	5	20
Ps. aeruginosa (43919)	50	50	50	Over 50

more rapidly as the inhibitory action of the drug declined. Therefore, the higher values reported by other investigators may be ascribed to their use of highly nutritive media, probably containing serum or blood, and to incubation for prolonged periods of time. The supposed direct inactivation of aureomycin by serum or blood reported by Chandler and Bliss[5] may have reflected an interplay of these factors rather than specific action on the drug.

The turbidimetric method with tryptose broth as the medium is probably the most accurate. It has the advantage of permitting rapid determinations, 3 or 4 hours of incubation being sufficient. It is more tedious and exacting than a simple plate method using tryptone agar, which may be preferable for routine laboratory use. Values obtained will be higher than those obtained by the turbidimetric method. Readings should be made within 12 hours of inoculation if possible since the error will be diminished with shorter periods of incubation. A correction factor may be employed, but this will vary widely with the experimental situation even at 12 hours. Eighteen hour determinations may be as much as 25 times greater than the equivalent 4 hour turbidimetric value.

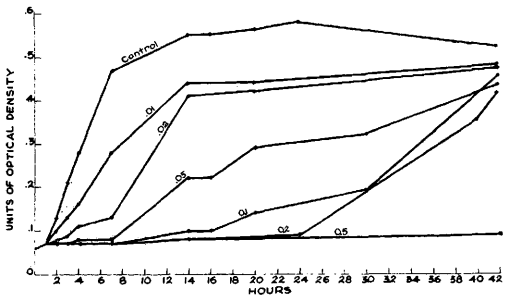

FIG. 1.
Staph. aureus growth curves.
(Aureomycin concentration in micrograms per ml).

Noting that bacterial sensitivity decreased as the size of the inoculum was increased, Harrell and associates[6] have warned of the potential danger of aureomycin resistance. This possibility has received further support from the detection of viable bacteria in the presence of very high concentrations of the agent. Such organisms were found on subsequent test to be fully sensitive as studied in this laboratory with the turbidimetric method. It is suggested that these bacteria were "persisters."

Summary. 1. Gram positive cocci and gram negative bacilli were tested turbidimetrically for aureomycin sensitivity. The inhibitory levels were .02 to .05 μg per ml for *S. aureus*, 0.1 μg per ml for enterococcus, and 0.5 μg per ml for *Sal. panama* and *E. coli*.

2. Aureomycin was not inhibited by the presence of blood or serum in the medium.

3. Increased length of incubation and addition of serum or blood to the medium resulted in higher levels of inhibition, particularly with the plate method. An explanation of this effect is offered.

4. Tryptone glucose extract agar was used for a plate method. Values were higher and less consistent than with the turbidimetric technic

5 Chandler, C. A., and Bliss, E. A., *Ann. New York Acad. Sci.*, 1948, **51**, 221.

6 Harrell, G. T., Meads, M., and Stevens, K., *South. Med. J.*, 1949, **42**, 4.

Received July 28, 1949. P.S.E.B.M., 1949, **72**.